Chapter 8

General discussion

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Protein secretion is an essential process for bacterial growth and a highly conserved process among bacterial species. It is also recognised as an important feature that can be widely used by industry for the commercial production of useful proteins, such as enzymes. Due to its GRAS status and its ability to secrete high amounts of proteins to the extracellular environment, the Gram-positive bacterium *Bacillus subtilis* is commercially exploited and often referred to as a "cell factory" for the production and secretion of a wide variety of proteins (Harwood, 1992). However, the secretion of heterologous proteins is often inefficient or not successful, due to particular properties of the secreted protein and/or the secretion machinery, or due to extracellular protein degradation (van Dijl *et al.*, 2002;Bolhuis *et al.*, 1999). The complete process of protein secretion - from production to translocation - is an extremely complex but organised mechanism of which we try to understand the details to overcome these bottlenecks. Patterns are discovered and models are being created in an effort to direct the utilisation of various protein secretion machineries *in vivo* as efficiently as possible.

Many routes for the translocation of proteins across membranes have been adopted by bacteria, which can vary enormously between different species. The most commonly used route for protein translocation in bacteria is the general secretion pathway (Sec), which translocates proteins in an unfolded manner. Parallel to the Sec pathway, the Twin-arginine translocation (Tat) pathway functions specifically for the secretion of folded and/or cofactor- containing proteins and forms the focus of this thesis.

The utilisation of the Tat pathway for heterologous protein secretion by *B. subtilis*

Presenting potential for translocation of pre-folded proteins across the membrane, heterologous protein secretion directed *via* the Tat pathway may possibly evade several bottlenecks, such as extracellular protein degradation. Indeed, experimental evidence is available for efficient secretion of Sec substrates *via* the Tat pathway, after their fusion to a Tat signal peptide. Subtilisin of *B. subtilis* was efficiently secreted *via* the TatAyCy machinery when the former was fused to the signal peptide of the TatAyCy substrate YwbN (Kolkman *et al.*, 2008). In addition, *E. coli* phytase AppA fused to the signal peptide of PhoD could be successfully translocated by the TatAdCd translocase in *B. subtilis* (Gerlach *et al.*, 2004). However, the requirements for heterologous protein secretion *via* the Tat pathway in *B. subtilis* are still far from understood. In contrast to these few success stories, the majority of tested proteins of interest could not be directed and secreted *via* Tat, or resulted in mixed observations, depending on the Tat signal peptide used (Kolkman *et al.*, 2008). Even though the presence of an active Tat translocase and a Tat signal peptide are vital requirements, additional essential factors include: a) specific properties of the

mature protein (McDonough *et al.*, 2008), b) correct protein folding within the cytoplasm (DeLisa *et al.*, 2003), c) the availability of chaperones or folding catalysts (Maillard *et al.*, 2007;Palmer *et al.*, 2005;Jack *et al.*, 2004;Oresnik *et al.*, 2001) and d) an energy source (Alder and Theg, 2003a). An overview of these and other requirements is shown in Figure 1.

Tat-dependent protein secretion in B. subtilis – one for each instead of one for all

The chromosome of *B. subtilis* encodes three *tatA* genes and two *tatC* genes, of which the products of *tatAd* and *tatCd* form an active complex together, as well as TatAy and TatCy. Interestingly, both Tat complexes are responsible for the secretion of one specific substrate: PhoD is secreted *via* TatAdCd, whereas YwbN is secreted *via* TatAyCy. Even though 69 additional proteins are included in a list of putative Tat-dependent substrates due to an RR or KR motif in their signal peptides (Jongbloed *et al.*, 2002), only two of these (QcrA and YkuE) have so far tested positive in a Tat-dependent reporter system in *Streptomyces* (Widdick *et al.*, 2008, Addendum of this thesis). Nevertheless, their preferred pathway for secretion in *B. subtilis* has not yet been determined. The difficulty to identify additional Tat substrates in *B. subtilis*, by 2D PAGE analysis (Jongbloed *et al.*, 2002;Jongbloed *et al.*, 2000) or Tat reporter systems, implicates that even though *B. subtilis* has two Tat translocases, they are responsible for a very small and specific subset of substrates. This is in high contrast to other organisms, for which a much larger number of Tat substrates has been identified (Tullman-Ercek *et al.*, 2007;Widdick *et al.*, 2006). Such an observation immediately raises questions like "*why are there at least two translocases for such a limited number of substrates?*", "*why are specifically PhoD and YwbN translocated via the Tat pathway?*" and most importantly "*what determines the specificity in Tat-dependent protein translocation in B. subtilis?*".

TAT – Translocation After Trigger?

When taking a closer look at the two identified Tat substrates of *B. subtilis*, clues can be obtained to eventually answer the first two questions addressed above. It is apparent that both substrates are produced in response to environmental stress and play a role in the cell's survival mechanisms directed towards that stress. PhoD is a phosphodiesterase directed towards the cell wall and secreted into the extracellular medium to liberate phosphate from complex phosphate-containing molecules. The transcription of the *phoD* operon is activated under low phosphate growth conditions. Interestingly, PhoD is a possible moonlighting protein as its expression in rich medium induces the transcription of the *tatAd* and *tatCd* genes (Chapter 6), which were previously thought to be expressed in response to low amounts of phosphate only (Eder *et al.*, 1996). However, the reason why PhoD is Sec-incompatible and is translocated *via* the Tat pathway is unknown. A possible explanation could be pre-translocational folding due to cofactor binding.
YwbN is a predicted iron-dependent peroxidase (Sturm *et al.*, 2006) and is secreted *via* the TatAyCy pathway of *B. subtilis*. Its proposed function strongly suggests its ability to bind ferric cofactors, which may be the reason why YwbN is translocated *via* the Tat pathway.

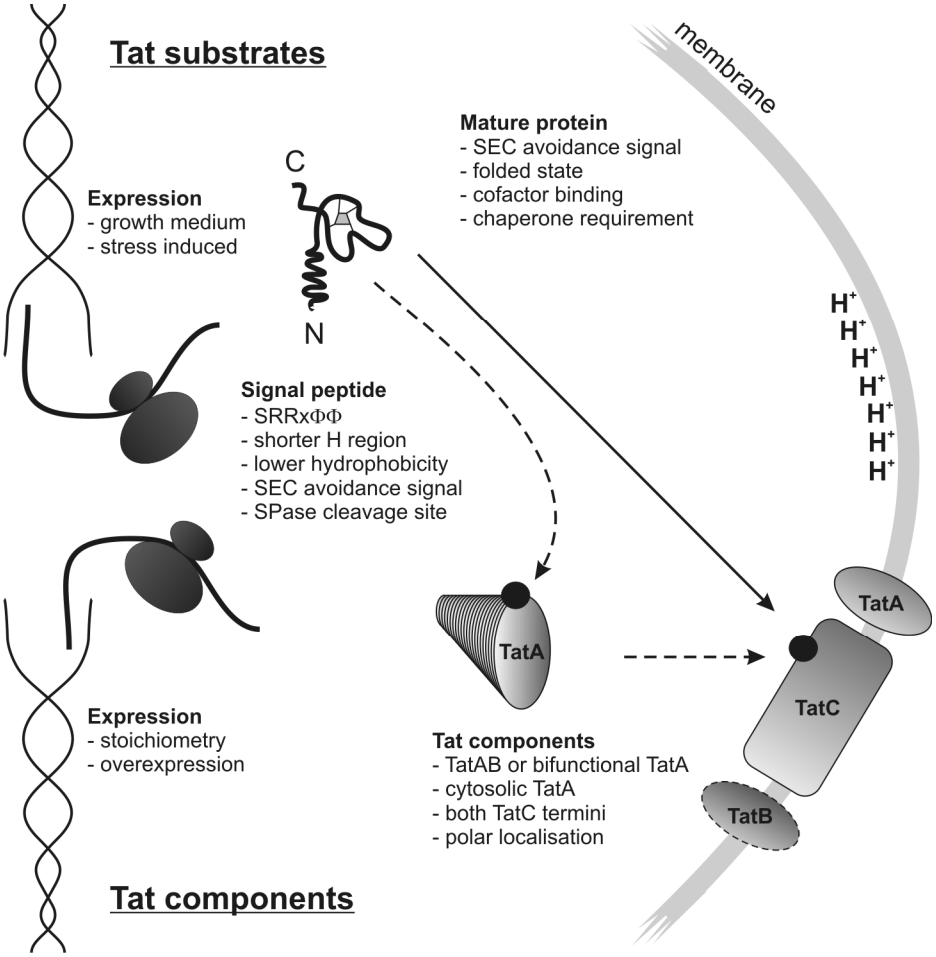

Figure 1 **General specificity determinants for Tat-dependent protein translocation.** The expression of Tat substrates and chaperones depends on the composition of the growth medium and/or stress-induced conditions. These have an effect on the availability of cofactors that are required for proper substrate folding. The signal peptide is a major specificity determinant and will (mostly) contain the highly conserved RR dipeptide and additional determining properties, such as a shorter and less hydrophobic H region and a Sec-avoidance signal in the C region, close to the signal peptidase (SPase) cleavage site. The mature protein also contains specificity determinants, possibly in the form of Sec-avoidance charges. The folded state of the protein is crucial and can depend on the binding of cofactors and the availability of chaperones. The stoichiometry of the individual Tat components may affect the functionality of the actual complex or the degree of specificity displayed by the complex. A functional Tat complex consists of TatC with a bifunctional TatA component (Gram-positive bacteria), or TatA and TatB components (Gram-negative bacteria and chloroplasts). Both termini of TatC (the initial substrate recognition- and binding site) are essential for functionality. Additionally, a cytosolic TatA complex may be present and function in the translocation process. Substrates can interact with the cytosolic TatA complex before being transfered to the membrane-bound TatA(B)C complex (dashed arrow), or interact directly with membrane-bound Tat(B)C (solid arrow). An energy source in the form of a proton gradient (H^+) and/or proton motive force is essential for efficient substrate translocation.

Its gene is located in the *ywbLMNO* operon, of which the expression is required during growth of *B. subtilis* in low iron minimal medium lacking citric acid (Ollinger *et al.*, 2006). During these conditions, YwbN presumably forms part of a YwbLMN elemental iron uptake system (Ollinger *et al.*, 2006).
The roles of the two known Tat substrates in stress-related growth conditions imply that the secretion of these proteins by the Tat machinery may be a response to specific triggers in the environment. This hypothesis is further supported by the identification of two additional putative Tat substrates, QcrA and YkuE (Widdick *et al.*, 2008, Addendum of this thesis). QcrA is a cytochrome c oxidoreductase similar to Rieske-type iron-sulfur proteins. Even though the preferred (Tat) pathway for QcrA has not yet been identified in *B. subtilis*, its dependency on the Tat pathway for secretion is very likely, as multiple previous reports have described Tat-dependent secretion of Rieske-type iron-sulfur proteins in various organisms (Aldridge *et al.*, 2008;De Buck *et al.*, 2007;Bachmann *et al.*, 2006;Molik *et al.*, 2001). Transcription of the *qcr* operon in *B. subtilis* is induced at the end of the exponential growth phase (Yu *et al.*, 1995) when the cells enter the transition phase and start to adapt to limiting nutrients in the environment. The function of the other putative Tat substrate, YkuE, is unknown, although its gene is not expressed during growth in a rich medium (Ridder and Blom, manuscript in preparation) and is probably induced during specific growth conditions, possibly in relation to environmental stress.
Finally, the third TatA protein of *B. subtilis*, TatAc, does not yet have an assigned function. It has been established that it does not play a role in the secretion of PhoD or YwbN (Eijlander *et al.*, 2009, Chapter 4 of this thesis;Jongbloed *et al.*, 2004), although a function in Tat-dependent protein translocation cannot be excluded. It is possible that production of TatAc proteins during specific growth conditions provides an additional pool of TatA components dedicated to the translocation of a specific, yet to be identified, substrate (in cooperation with TatCd or TatCy).

The role of individual Tat components

When Sec-independent protein secretion was first observed during the early 1990's in thylakoids of chloroplasts, three proteins were quickly acknowledged to make up a unique pathway, able to translocate folded proteins independent of ATP for energy. Homologues of TatA, TatB and TatC were subsequently identified in a wide variety of organisms, although some groups of bacteria, such as lactic acid bacteria, are devoid of any *tat* genes.
Extensive research directed towards the understanding of protein translocation *via* this newly discovered pathway picked up rapidly. Even though many apparent similarities are present, there are also some conspicuous differences. The presence of multiple, separate Tat translocases, or the number of substrates dependent on the Tat pathway for their secretion can differ enormously between species. Examples of other differences are illustrated by the results described in Chapters 2 and 3. For instance, the TatAdCd and TatAyCy complexes of *B. subtilis* are significantly smaller in size when compared to the TatABC complex of *E. coli*. Furthermore, the organisation of the Gram-positive TatA and TatAC complexes also proves to be more homogeneous when compared to *E. coli* TatA and TatABC. Interestingly, these distinct differences do not seem to have implications on the working

mechanism of substrate recognition and/or translocation, since both Tat complexes of *B. subtilis* are active in *E. coli* when heterologously expressed (Chapters 2 and 3).

TatA – efficient multi-tasking or slightly 'schizophrenic'?

One of the most striking differences between Gram-negative and Gram-positive Tat systems is the absence of the TatB component from all Gram-positive bacteria, with the exception of actinomycetes species, such as *Streptomyces*. Even though this protein is essential for Tat-dependent protein translocation in *E. coli* (Sargent *et al.*, 1999), *B. subtilis* and many other Gram-positives seem to do very well without it. The TatA and TatB proteins show quite some similarities, both on the level of amino acid sequence and on predicted topology in the membrane. Nevertheless, each have a specific function in Tat complex formation and the substrate translocation process (Orriss *et al.*, 2007;Mangels *et al.*, 2005;De Keersmaeker *et al.*, 2005b;Sargent *et al.*, 1999). It has therefore been proposed that Gram-positive TatA may be bifunctional; a feature that was experimentally proven in a study described in Chapter 2. Overproduction of *B. subtilis* TatAd in a Δ*tatA/E* and Δ*tatB* background of *E. coli* resulted in the secretion of the TorA Tat substrate, indicating that TatAd is in fact bifunctional and able to execute the functions of both *E. coli* TatA and TatB (Barnett *et al.*, 2008). Previous studies, involving genetically modified *E. coli* TatA proteins able to complement the absence of both TatA/E and TatB, already provided essential clues on the possible bifunctional character of the TatA protein (Blaudeck *et al.*, 2005).

The TatA protein forms part of the structural TatABC unit, but also forms a separate homooligomeric complex in the membrane (Barnett *et al.*, 2009;Barnett *et al.*, 2008;Oates *et al.*, 2005). Single-particle electron microscopy analysis of TatA revealed a ring-shaped structure, possibly representing a pore through which protein translocation can occur (Gohlke *et al.*, 2005). This hypothesis was recently debated in a quantitative study describing the TatA complex in *Arabidopsis thaliana* (Jakob *et al.*, 2009), in which the authors favour a different model in which TatA facilitates Tat-dependent protein transport by "weakening" the membrane in a yet unknown manner. Such weakening might enable the substrates to pass the lipid phase either directly or by assistance of a conformational change of the TatB/C receptor complex, as was previously suggested by Natale *et al*. (2008).
Next to its membrane-associated localisation (Porcelli *et al.*, 2002), several studies in a variety of organisms have also described the presence and functionality of separate, cytosolic TatA complexes (Berthelmann *et al.*, 2008;Frielingsdorf *et al.*, 2008;Westermann *et al.*, 2006;De Keersmaeker *et al.*, 2005a;Pop *et al.*, 2003). In *B. subtilis*, soluble TatAd molecules were shown to specifically interact with the signal peptide of PhoD, and are thought to function as the initial recognition and binding site for the PhoD substrate before targeting to and translocation by a membrane-bound TatAdCd complex (Westermann *et al.*, 2006;Pop *et al.*, 2003). For chloroplasts, a soluble form of TatA in the stroma has been described, capable of triggering the membrane translocation step during Tat-dependent protein transport (Frielingsdorf *et al.*, 2008). Furthermore, in *E. coli* tubular structures of

soluble TatA modules have been observed alongside membrane-localised TatA (Berthelmann *et al.*, 2008), possibly functioning as targeting factors for substrates.
Even though a distinct function of soluble TatA molecules and complexes has not yet been defined, and the possibility of observed artifacts due to overexpression of the Tat components still exists, the above described observations indicate an extensive role of the TatA component in Tat-dependent protein translocation and highlight detailed differences between the Tat translocases of individual organisms that may in fact play a determining role in substrate specificity (illustrated in Figure 1).

TatC – the Tat complex 'gatekeeper'

Of the identified Tat proteins, TatC is the largest with six transmembrane spanning domains and an experimentally proven N-in - C-in topology (Punginelli *et al.*, 2007;Behrendt *et al.*, 2004). Together with TatB it forms the initial Tat substrate receptor complex and is known to interact specifically with the RR consensus region in the substrate signal peptide (Alami *et al.*, 2003). Additionally, several other functions have been assigned to TatC, indicating that this protein plays a vital role in the substrate translocation process. In *E. coli*, TatC is required for controlling the oligomeric state of TatA (Leake *et al.*, 2008) and is also involved in the formation of tubular TatA structures and the polar localisation of the Tat components (Berthelmann *et al.*, 2008;Berthelmann and Brüser, 2004). TatC drives the assembly of Tat complexes in both *E. coli* and *Streptomyces* species (De Keersmaeker *et al.*, 2007;Mangels *et al.*, 2005) and is even essential for viability in halophilic archaea (Thomas and Bolhuis, 2006).
The TatC protein sequence is not very conserved among Tat-containing organisms, although certain regions in TatC contain a few highly conserved residues. The role and importance of these residues, as well as less conserved ones, has been studied extensively in Chapter 5 of this thesis. Because TatC has been assigned multiple functions, it is feasible that multiple regions of TatC are essential for efficient Tat substrate translocation. This is clearly demonstrated by the construction and analysis of several TatCd/TatCy protein chimera of *B. subtilis*, which were all inactive in protein translocation (Chapter 5). Nevertheless, multiple observations from several independent studies indicate that especially the N-terminal half of TatC is involved in Tat substrate recognition and binding (Holzapfel *et al.*, 2007;McDevitt *et al.*, 2006;Buchanan *et al.*, 2002), with a possible role of the C terminus in complex formation and stability (Chapter 5).
Interestingly, modification of several conserved amino acids has different effects on the protein translocation efficiency, often depending on the organism or the Tat substrate at study. Even within the same organism the importance of conserved residues in two paralogous TatC proteins can differ, as is illustrated by the mutagenesis study described for the first two cytoplasmic regions of *B. subtilis* TatCd and TatCy (Chapter 5). Because only two Tat substrates for *B. subtilis* have been identified to date, and both rely on a different pathway for their translocation, it is a challenge to test the importance of conserved residues between the two TatC proteins without additional effects caused by the use of different substrates. To conclude whether the observed differences in effects are caused by specific Tat substrate properties or are in fact due to a different working mechanism displayed by

the two Tat complexes of *B. subtilis*, the translocation efficiency by the modified TatC proteins should be studied using the same substrate.

The role of the Tat signal peptide

Almost all proteins directed *via* the Tat pathway have a common feature in the N-terminal region of their signal peptide: the double arginine residues. The importance of these highly conserved arginines has been studied considerably, which has resulted in mixed observations, often depending on the organism or substrate at study. In chloroplasts, substitution of either arginine, even by lysine, results in a complete block in translocation by the thylakoidal Tat system (Henry *et al.*, 1997;Chaddock *et al.*, 1995). In *E. coli* however, replacement of one of the arginines by a lysine still allows for translocation, albeit less efficiently than two consecutive arginines, whereas substitution of both arginines blocks translocation completely (Stanley *et al.*, 2000). The same applies for substrates translocated *via* the TatAdCd pathway of *B. subtilis* (Mendel *et al.*, 2008, Chapter 3 of this thesis). In addition, some naturally occurring Tat substrates without the typical RR motif in the signal peptide have also been described (McDonough *et al.*, 2008;Ignatova *et al.*, 2002;Hinsley *et al.*, 2001). The fact that these double arginines play an important role, but are not solely responsible for Tat-directed translocation is further demonstrated by the observation that substitutions of the double arginines by lysines, or even alanines, in the *E. coli* SufI substrate lead to a complete block of translocation, but have no effect on substrate binding by the TatBC complex (McDevitt *et al.*, 2006).

Protein translocation via the TAT pathway – there's more to it than just T(h)AT

Even though a large list of putative Tat substrates for a variety of organisms can be drawn up based on the specific double arginine peptide, in practice, the majority of these proteins turn out to be false positives (Widdick *et al.*, 2008, Addendum of this thesis). This could be due to the fact that additional features in the proximity of the RR consensus motif have been identified as being equally important (Brink *et al.*, 1998; also see Figure 1). The role of three of such conserved residues is described in Chapter 3. Next to the highly conserved double arginine residues, the serine directly in front of the double arginines is extremely sensitive to modification, although the degree of sensitivity is evidently substrate-specific (Mendel *et al.*, 2008). Furthermore, hydrophobic residues on the +2 and +3 positions of the SPase cleavage site play a key role in Tat-dependent protein translocation, of which the phenylalanine on position +2 and the leucine on position +3 in *E. coli* Tat signal peptides have been studied as described in Chapter 3.

The hydrophobic region (H region) of Tat signal peptides is also acknowledged to play a determining role in Tat-dependent protein export. Generally, in Tat signal peptides the H region is shorter (Brink *et al.*, 1997) and has a lower hydrophobicity (Cristóbal *et al.*, 1999) when compared to Sec signal peptides. Furthermore, the signal peptide of the *E. coli* Tat substrate TorA contains a leucine-rich region in the H domain that apparently plays a critical role in the binding of the TorD 'Tat proofreading' chaperone (Buchanan *et al.*,

2008), which is an essential step for cofactor loading (Pommier *et al.*, 1998) and correct TorA folding and targeting (Jack *et al.*, 2004).
Likewise, the C region of the Tat signal peptide does not only contain the signal peptidase cleavage site, but also plays an important role in the Tat-dependent character of the substrate. Earlier reports already suggested that the presence of a positive charge in the C region serves as a Sec-avoidance signal (Bogsch *et al.*, 1997). The importance of this charge was later extended to the role of the first few amino acids of the mature protein in Sec avoidance (Tullman-Ercek *et al.*, 2007).

High definition structures of Tat components and complexes

The availability of high definition structures of the individual Tat components and Tat complexes will provide vital information for the understanding of the working mechanism of the Tat machinery. Studying the Tat proteins with electron microscopy and crystallography techniques can reveal new details on Tat component stoichiometry and Tat machine morphology, but also on putative substrate binding pockets or Tat component interaction sites. So far, no high definition structures of Tat complexes or individual components have been obtained, although some single-particle electron microscopy images have already provided clues on the 3D structure of the *E. coli* TatA complex, supporting the pore-forming theory (Gohlke *et al.*, 2005) and showing the formation of tube-like structures in the cytosol (Berthelmann *et al.*, 2008). In addition, solid state NMR has been applied to study the topology and orientation of *B. subtilis* TatAd (Müller *et al.*, 2007), followed by additional spectroscopy techniques to further analyse the conformations and alignments of TatAd fragments (Lange *et al.*, 2007). These studies revealed a helical membrane-spanning N-terminal region of TatAd and a helical cytoplasmic segment of TatAd aligned parallel to the bilayer surface. Furthermore, photophysical analysis with additional *in silico* data analysis was applied to study the oligomeric state of TatA in *E. coli* (Leake *et al.*, 2008). Such techniques have already provided a large amount of data concerning the number of Tat subunits per complex and the stoichiometry of these subunits when high resolution structural images are not yet available.

Tat-dependent protein secretion in pathogens

The proteins making up the Tat machinery have also been identified for a wide range of pathogenic bacteria. The role of Tat in the virulence of these organisms is an important subject of study from a medical and pharmaceutical point of view. In the last few years the number of reports documenting a role of the Tat pathway in virulence has increased significantly (reviewed in De Buck *et al.*, 2008b). Deletion mutants of *tat* genes have for instance resulted in affected phenotypes and attenuated virulence for *Pseudomonas* species (Voulhoux *et al.*, 2006;Caldelari *et al.*, 2006;Bronstein *et al.*, 2005;Ochsner *et al.*, 2002), *Yersinia* species (reviewed in Lavander *et al.*, 2007), *Vibrio fischeri* (Dunn and Stabb, 2008) and *Legionella pneumophila* (De Buck *et al.*, 2008a;De Buck *et al.*, 2005). The effects of the absence of *tat* genes depend on the organism or strain at study, but mostly include loss of motility, increased sensitivity to environmental stress, decreased biofilm formation, decreased iron-acquisition, retarded cell growth and affected cell wall integrity. The direct effect of a *tatC* mutant of *Pseudomonas aeroginosa* on virulence has been tested

in rat lung models, which remained lesion-free, highlighting the importance of the Tat pathway for virulence (Ochsner *et al.*, 2002). In *Mycobacterium tuberculosis* it has not been possible to construct *tat* mutants, indicating that the Tat pathway is essential for the viability, and possibly pathogenicity, of this organism (Saint-Joanis *et al.*, 2006). The presence of a functional Tat pathway has also been described for a number of other pathogenic bacteria, such as *Helicobacter pylori, Vibrio cholerae* and *Staphylococcus aureus*, although its role in virulence has not yet been determined.

TAT – Tomorrow's Anti-microbial Target?

Because the Tat pathway has been shown to play an important role in the virulent character of several pathogenic bacteria, it may form an interesting target for antimicrobial therapeutics. However, similar to what was previously described for Tat-containing non-pathogenic bacteria, a clear species-, strain- and substrate specificity of the Tat system is observed in pathogenic species as well. Homologues of the same substrate protein are either secreted *via* the Sec pathway or *via* the Tat pathway, depending on the bacterial strain (Gonzalez *et al.*, 2007). It has therefore been suggested that the Tat pathway in some strains has been adapted to serve the particular needs of the pathogen (De Buck *et al.*, 2008b), indicating a highly specialised mechanism that will be difficult to target with general antimicrobials. This is also illustrated by a recent study by McDonough *et al*. (2008), in which the existence of *M. tuberculosis*-specific factors for Tat export of established virulence proteins is described, that were not identified for the Tat-containing non-pathogenic *M. smegmatis* species.
Also, the Tat pathway is localised in the inner membrane, creating the requirement for the antimicrobial compound to cross the outer membrane of Gram-negative bacteria, or the thick peptidoglycan layers of the cell wall of Gram-positive bacteria. And with the exception of *M. tuberculosis*, the Tat system is not essential for viability, eliminating the option to effectively kill the bacterial pathogens. Finally, the *tat*-deficient pathogens may contain alternative routes for the secretion of virulence factors, resulting in an attenuation, but not complete loss of virulence. Taken together, these limitations do not yet make the Tat pathway a more favourable target for antimicrobial compounds than, for instance, the more general Sec pathway.

Conclusion and future directions

Before efficient utilisation of the Tat complex of *B. subtilis* and other Gram-positive species in heterologous protein secretion or as an antimicrobial target can be realised, we need to answer the question: '*what determines the specificity in Tat-dependent protein translocation?*'. Therefore, it is important to study the requirements of efficient translocation of Tat substrates during specific growth conditions (Chapter 6 and Addendum of this thesis). The results described in this thesis, and conclusions drawn from these results, strongly suggest that models created for Tat-dependent protein secretion in thylakoids and *E. coli* cannot *per se* be applied to *B. subtilis*. Significant differences have

been observed in the working mechanism and substrate specificity between the Tat translocases of *E. coli* and *B. subtilis* (Chapters 2, 3 and 5 of this thesis) and even between the two Tat translocases of *B. subtilis* (TatAdCd and TatAyCy) (Chapter 5 of this thesis). Also, the fact that the substrate specificity can be relaxed during certain expression conditions should be considered: when present in excess amounts, the TatAdCd complex is able to translocate a wide variety of Tat-dependent substrates (Chapters 2 and 3 of this thesis), including TatAyCy-dependent YwbN (Chapter 4 of this thesis). Possibly the former translocase is therefore a more apt candidate for heterologous protein secretion than the TatAyCy complex.

The substrate specificity displayed by both Tat complexes of *B. subtilis* is clearly the result of a combination of specific properties of the TatA and TatC subunits, as well as the signal peptide and possibly also the mature protein of the substrate involved (Figure 1). Therefore, more detailed genetic and biochemical studies are required to determine the exact mode of action and the requirements for efficient protein translocation by either complex. Such studies should focus on the identification of additional substrates, the Tat complex formation requirements and also Tat substrate recognition requirements. Detailed mutagenesis studies, involving the TatA and TatC components, as well as the substrate signal sequences and N-terminal mature sequence, combined with structural and biochemical protein-protein interaction studies will offer new insights on specific Tat-substrate interaction requirements. In the future, all combined obtained data can be used for the construction of a Tat signal peptide- and Tat component library to eventually identify the optimal signal peptide, combined with the optimal Tat translocase for efficient Tat-dependent translocation of proteins of interest. At first, a case by case approach is inevitable (because Tat-dependent protein translocation in *B. subtilis* depends on many different factors) in which a few basic requirements should always be taken into account. Firstly, determining the folding characteristics of the substrate of interest (*i.e.* cofactor binding and chaperone requirement) is crucial. Secondly, to ensure Tat-dependent secretion and to prevent secretion of malfolded proteins, a Sec-avoidance signal in the form of a positive charge at the C region (including a short region of the mature protein in some cases) as well as an optimal RR consensus sequence in the N region of the signal peptide are equally essential. Finally, increased production of Tat(Ad and Cd) components can result in a more relaxed substrate specificity and facilitate the secretion of (heterologous) proteins.